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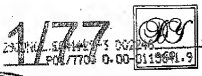

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   Patents ADP number *(if you know it)* 05806244001

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5. Name of your agent *(if you have one)* — D Young & Co

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Signature: Louise Holliday
D YOUNG & CO
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Date: 28 Jun 2001

12. Name and daytime telephone number of person to contact in the United Kingdom: Antonio Maschio 023 8071 9500

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Ligand

The present invention relates to a method for the preparation of dual-specific ligands comprising a first single immunoglobulin variable domain region binding to a first antigen, and a second complementary immunoglobulin single variable domain region binding to a second antigen. Dual-specific ligands and their uses are also described.

Introduction

The antigen binding domain of an antibody comprises two separate regions: a heavy chain variable domain (V_H) and a light chain variable domain (V_L; which can be either V_κ or V_λ). The antigen binding site itself is formed by six polypeptide loops: three from V_H domain (H1, H2 and H3) and three from V_L domain (L1, L2 and L3). A diverse primary repertoire of V genes that encode the V_H and V_L domains is produced by the combinatorial rearrangement of gene segments. The V_H gene is produced by the recombination of three gene segments, V_H, D and J_H. In humans, there are approximately 51 functional V_H segments (Cook and Tomlinson (1995) *Immunol Today*, **16**: 237), 25 functional D segments (Corbett *et al.* (1997) *J. Mol. Biol.*, **268**: 69) and 6 functional J_H segments (Ravetch *et al.* (1981) *Cell*, **27**: 583), depending on the haplotype. The V_H segment encodes the region of the polypeptide chain which forms the first and second antigen binding loops of the V_H domain (H1 and H2), whilst the V_H, D and J_H segments combine to form the third antigen binding loop of the V_H domain (H3). The V_L gene is produced by the recombination of only two gene segments, V_L and J_L. In humans, there are approximately 40 functional V_κ segments (Schäble and Zachau (1993) *Biol. Chem. Hoppe-Seyler*, **374**: 1001), 31 functional V_λ segments (Williams *et al.* (1996) *J. Mol. Biol.*, **264**: 220; Kawasaki *et al.* (1997) *Genome Res.*, **7**: 250), 5 functional J_κ segments (Hieter *et al.* (1982) *J. Biol. Chem.*, **257**: 1516) and 4 functional J_λ segments (Vasicek and Leder (1990) *J. Exp. Med.*, **172**: 609), depending on the haplotype. The V_L segment encodes the region of the polypeptide chain which forms the first and second antigen binding loops of the V_L domain (L1 and L2), whilst the V_L and J_L segments

combine to form the third antigen binding loop of the V_L domain (L3). Antibodies selected from this primary repertoire are believed to be sufficiently diverse to bind almost all antigens with at least moderate affinity. High affinity antibodies are produced by "affinity maturation" of the rearranged genes, in which point mutations are generated and selected by the immune system on the basis of improved binding.

Analysis of the structures and sequences of antibodies has shown that five of the six antigen binding loops (H1, H2, L1, L2, L3) possess a limited number of main-chain conformations or canonical structures (Chothia and Lesk (1987) *J. Mol. Biol.*, **196**: 901; Chothia *et al.* (1989) *Nature*, **342**: 877). The main-chain conformations are determined by (i) the length of the antigen binding loop, and (ii) particular residues, or types of residue, at certain key position in the antigen binding loop and the antibody framework. Analysis of the loop lengths and key residues has enabled us to the predict the main-chain conformations of H1, H2, L1, L2 and L3 encoded by the majority of human antibody sequences (Chothia *et al.* (1992) *J. Mol. Biol.*, **227**: 799; Tomlinson *et al.* (1995) *EMBO J.*, **14**: 4628; Williams *et al.* (1996) *J. Mol. Biol.*, **264**: 220). Although the H3 region is much more diverse in terms of sequence, length and structure (due to the use of D segments), it also forms a limited number of main-chain conformations for short loop lengths which depend on the length and the presence of particular residues, or types of residue, at key positions in the loop and the antibody framework (Martin *et al.* (1996) *J. Mol. Biol.*, **263**: 800; Shirai *et al.* (1996) *FEBS Letters*, **399**: 1.

Bispecific antibodies comprising complementary pairs of VH and VL regions are known in the art. Methods described involve hybrid hybridomas (Milstein & Cuello AC Nature 305:537-40), minibodies (Hu *et al.*, (1996) Cancer Res 56:3055-3061;), diabodies (Holliger *et al.*, (1993) Proc. Natl. Acad. Sci. USA 90, 6444-6448; WO 94/13804), chelating recombinant antibodies (CRAbs; (Neri *et al.*, (1995) J. Mol. Biol. 246, 367-373), biscFv (e.g. Atwell *et al.*, (1996) Mol. Immunol. 33, 1301-1312), "knobs in holes" stabilised antibodies (Carter *et al.*, (1997) Protein Sci. 6, 781-788). In each case each antibody species comprises two antigen-binding sites, each fashioned by a complementary pair of VH and VL domains. Each antibody is thereby able to bind to two different antigens at the same time. Each of these techniques presents its particular disadvantages; for instance in the case of hybrid hybridomas, inactive VH/VL pairs can

greatly reduce the fraction of bispecific IgG. In other cases, it is necessary to engineer the heavy or light chains at the sub-unit interfaces (Carter *et al.*, 1997).

There is some evidence that two different antibody binding specifities might be incorporated into the same binding site. For example, cross-reactive antibodies have been described, usually where the two antigens are related in sequence and structure, such as hen egg white lysozyme and turkey lysozyme ((**McCafferty et al., WO 92/01047**) or to free hapten and to hapten conjugated to carrier (Griffiths AD et al, *EMBO J* 1994 13:14 3245-60. Furthermore natural autoantibodies have been described that are polyreactive (Casali & Notkins, Ann. Rev. Immunol. 7, 515-531), reacting with at least two (usually more) different antigens that are not structurally related. It has also been shown that selections of random peptide repertoires using phage display technology on a monoclonal antibody will identify a range of peptide sequences that fit the antigen binding site. Some of the sequences are highly related, fitting a consensus sequence, whereas others are very different and have been termed mimotopes (Lane & Stephen, Current Opinion in Immunology, 1993, 5, 268-271). It is therefore clear that the binding site of an antibody, comprising associated and complementary VH and VL domains, has the potential to bind to many different antigens from a large universe of known antigens. It is less clear how to create a binding site to two given antigens, particularly those which are not necessarily structurally related.

Protein engineering methods have been suggested that may have a bearing on this. For example it has also been proposed that a catalytic antibody could be created with a binding activity to a metal ion through one variable domain, and to a hapten (substrate) through contacts with the metal ion and a complementary variable domain ((**Barbas et al., 1993 Proc. Natl. Acad. Sci USA 90, 6385-6389**).However in this case, the binding and catalysis of the substrate (first antigen) is proposed to require the binding of the metal ion (second antigen).

Methods have been described for the creation of bispecific antibodies from camel antibody heavy chain single domains in which binding contacts for one antigen are created in one variable domain, and for a second antigen in a second variable domain. However the variable domains were not complementary. Thus a first heavy chain variable domain is selected against a first antigen, and a second heavy chain variable domain

against a second antigen, and then both domains are linked together on the same chain to give a bispecific antibody fragment (Conrath et al., J. Biol. Chem. 270, 27589-27594). However the camel heavy chain single domains are unusual in that they are derived from natural camel antibodies which have no light chains, and indeed the heavy chain single domains are unable to associate with camel light chains to form complementary VH and VL pairs

Single heavy chain variable domains have also been described, derived from natural antibodies which are normally associated with light chains (from monoclonal antibodies or from repertoires of domains EP-A-0368684). It was suggested to make bispecific antibody fragments by linking heavy chain variable domains of different specificity together (as described above). The disadvantage with this approach is that isolated antibody variable domains have a hydrophobic interface that normally makes interactions with the light chain and is exposed to solvent and may be "sticky" allowing the single domain to bind to hydrophobic surfaces. Furthermore heavy and light chain variable domains that are not associated with a complementary light or heavy chain variable domain respectively may be less stable and readily unfold (Worn & Pluckthun, 1998 Biochemistry 37, 13120-7).

Summary of the invention

The inventors have realised that it is desirable to make bispecific antibodies in which the binding of a first antigen does not necessarily facilitate the binding of a second antigen. They have also realised that the solution lies in creating binding contacts for the first antigen in one variable domain, and binding contacts for the second antigen in the complementary variable domain, and that further significant advantages over the bispecific antibodies of the prior art may be derived by bringing together complementary single variable domains of differing specificities; for example, a heavy chain variable domain that binds to a first antigen with a light chain variable domain that binds to a second antigen. Thus each VH/VL pair has two binding specificities. These combinations of domains are referred to as 'dual-specific' ligands.

The inventors have found that the use of complementary variable domains allows the two domain surfaces to pack together and be sequestered from the solvent. Furthermore the

complementary domains are able to stabilise each other. In addition, it allows the creation of dual-specific IgG antibodies without the disadvantages of hybrid hybridomas previously discussed, or the need to engineer heavy or light chains at the sub-unit interfaces. The dual-specific ligands of the present invention have at least one VH/VL pair. A bispecific IgG will therefore comprise two such pairs, one pair on each arm of the Y-shaped molecule.

In a first aspect, therefore, the present invention provides a method for producing a dual-specific ligand comprising a first single immunoglobulin variable domain having a first binding specificity and a complementary immunoglobulin single variable domain having a second binding specificity, the method comprising the steps of:

(a) selecting a first variable domain by its ability to bind to a first antigen,
(b) selecting a second variable region by its ability to bind to a second antigen,
(c) combining the variable regions; and
(d) selecting the dual-specific ligand by its ability to bind to said first and second antigens.

In a preferred embodiment of the invention each single variable domain may be selected for binding to its target antigen in the absence of a complementary variable region. In an alternative embodiment, the single variable domains may be selected for binding to its target antigen in the presence of a complementary variable region. Thus the first single variable domain may be selected in the presence of a third complementary variable domain, and the second variable domain may be selected in the presence of a fourth complementary variable domain. In this case the binding activity of first (or second) variable domain may not be evident except in the presence of the complementary third (or fourth) variable domain. The complementary third or fourth variable domain may be the natural cognate variable domain having the same specificity as the single domain being tested, or a non-cognate complementary domain – such as a "dummy" variable domain.

Advantageously, the single variable domains are derived from antibodies selected for binding activity against different antigens.

Preferably, the dual specific ligand of the invention comprises only two complementary variable domains although several such ligands may be incorporated together into the same protein, for example two such ligands can be incorporated into an IgG.

It will be appreciated by one skilled in the art that the light and heavy variable regions of a dual-specific ligand produced according to the method of the present invention may be on the same polypeptide chain, or alternatively, on different polypeptide chains. In the case that the variable regions are on different polypeptide chains, then they may be linked via a linker, generally a flexible linker (such as a polypeptide chain), a chemical linking group, or any other method known in the art.

The first and the second antigen binding domains may be associated either covalently or non-covalently. In the case that the domains are covalently associated, then the association may be mediated for example by disulphide bonds.

The first and the second antigens are different. They may be polypeptides, proteins or nucleic acids, which may be naturally occurring or synthetic. One skilled in the art will appreciate that the choice of antigen is many and varied. They may be for instance human or animal proteins, enzymes, co-factors for enzymes or DNA binding proteins. It will be appreciated that this list is by no means exhaustive.

The antigens may compete for binding to the dual-specific ligand, such that they may not both bind simultaneously. Alternatively, they may both bind simultaneously, such that the dual-specific ligand bridges the antigens.

In one embodiment of the invention, the variable domains are derived from an antibody directed against the first and/or second antigen. In a preferred embodiment the variable domains are derived from a repertoire of single antibody domains.

In a second aspect, the present invention provides a dual-specific ligand comprising a first single immunoglobulin variable domain having a first binding specificity and a complementary immunoglobulin single variable domain having a second binding specificity.

Advantageously, the dual-specific ligand according to the second aspect of the invention is obtainable by the method of the first aspect of the present invention.

In a preferred embodiment of this aspect of the invention, the ligand comprises one single heavy chain variable domain of an antibody and one complementary single light chain variable domain of an antibody such that the two regions are capable of associating to form a complementary VH/VL pair.

A dual-specific ligand of this nature permits the two complementary variable region surfaces to pack together and be sequestered from the solvent and to help stabilise each other.

In a third aspect, the present invention provides one or more nucleic acid molecules encoding at least a dual-specific ligand as herein defined.

The nucleic acid may further encode a signal sequence for export of the polypeptides from a host cell upon expression and may be fused with a surface component of a filamentous bacteriophage particle (or other component of a selection display system) upon expression.

In a further aspect the present invention provides a vector comprising nucleic acid according to the present invention.

In a yet further aspect, the present invention provides a host cell transfected with a vector according to the present invention.

Expression from such a vector may be configured to produce, for example on the surface of a bacteriophage particle, variable domains for selection. This allows selection of displayed variable regions and thus selection of 'dual-specific ligands' using the method of the present invention.

The present invention further provides a kit comprising at least a dual-specific ligand according to the present invention.

Dual-specific ligands produced according to the present invention may comprise one arm of an IgG molecule, or comprise a single chain Fv fragment or comprise a Fab region.

In a preferred embodiment of the invention, the variable regions are selected from single domain V gene repertoires. Generally the repertoire of single antibody domains is displayed on the surface of filamentous bacteriophage. In a preferred embodiment each single antibody domain is selected by binding of a phage repertoire to antigen.

In a further aspect, the present invention provides a composition comprising an dual-specific ligand or a fragment thereof, obtainable by a method of the present invention, and a pharmaceutically acceptable carrier, diluent or excipient.

Moreover, the present invention provides a method for the treatment of disease using a 'dual-specific ligand' or a composition according to the present invention.

In a preferred embodiment of the invention the disease is cancer. For instance a 'bridging' dual specific ligand may be used to recruit cytotoxic T-cells to a cancer marker, or to bind to two different antigens on the surface of a cancer cell, thereby increasing the affinity or specificity of binding to the cell surface. For a complete IgG, comprised of bridging dual specific ligands, the antibody would be capable of binding to four molecules of antigen. Alternatively if the binding of one antigen displaces the other, such antibodies might be used to release a drug on binding of a cancer cell surface marker.

In a further aspect, the present invention provides a method for the diagnosis, including diagnosis of disease using a dual-specific ligand, or a composition according to the present invention. Thus binding of analyte (second antigen) could displace an enzyme (first antigen) bound to the antibody providing the basis for an immunoassay, especially if the enzyme were held to the antibody through its active site.

Brief Description of the Figures.

Figure 1 shows the diversification of VH/HSA at positions H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97, H98 (DVT or NNK encoded respectively) which are in the antigen binding site of VH HAS. The sequence of V_K is diversified at positions L50, L53.

Figure 2 shows Library 1: Germline V_K/DVT V_H,
Library 2: Germline V_K /NNK V_H,
Library 3: Germline V_H/DVT V_K
Library 4: Germline V_H/NNK V_K

In pIT2/ScFv format. These libraries were pre-selected for binding to generic ligands protein A and protein L so that the majority of the claims and selected libraries are functional. Libraries were selected on HSA (first round) and β-gal (second round) or HSA β-gal selection or on β-gal (first round) and HSA (second round) β-gal HSA selection. Soluble scFv from these claims of PCR are amplified in the sequence. One clone encoding a dual specific antibody K8 was chosen for further work.

Figure 3 shows an alignment of V_H chains and V_K chains.

Figure 4 shows the characterisation of the binding properties of the K8 antibody, the binding properties of the K8 antibody characterised by monoclonal faguliser, the dual specific K8 antibody was found to bind HSA and β-gal and displayed on the surface of the phage with absorbant signals greater than 1.0. No cross reactivity with other proteins was detected.

Figure 5 shows soluble scFv ELISA performed using known concentrated and some of the K8 antibody fragment. A 96-well plate was coated with 100μg of HSA, BSA and β-gal at 10μg/ml and 100μg/ml of Protein A at 1μg/ml concentration. 50μg of the serial dilutions of the K8 scFv was applied and the bound antibody fragments were detected with Protein L-HRP. ELISA results confirm the dual specific nature of the K8 antibody.

Figure 6 shows the binding characteristics of the clone K8V_K/dummy V_H analysed using soluble scFv ELISA. Production of the soluble scFv fragments was

induced by IPTG as described by Harrison *et al* and the supernatant containing scFv assayed directly. Soluble scFv ELISA is performed as described in example 1 and the bound scFvs were detected with Protein L-HRP. The ELISA results revealed that this clone was still able to bind β-gal, whereas binding BSA was abolished.

Figure 7 shows the binding of dual specific scFv antibodies directed against APS and β-gal and a dual specific scFv antibody directed against BCL10 protein and β-gal to their respective antigen.

Figure 8 shows the binding characteristics of $K8V_K/V_H2/K8V_K/V_H4$ and $K8V_K/V_HC11$ using a soluble scFv ELISA as described herein. All claims were bound to be dual specific without any cross-reactivity with other proteins.

Figure 9 shows the binding characteristics of produced clones V_H2sd and V_H4sd tested by monoclonal phage ELISA. Phage particles were produced as described by Harrison *et al* in 1996. 96-well ELISA plates were coated with 100μg/ml of APS, BSA, HSA, β-gal, ubiquitin, α-amylase and myosin at 10μg/ml concentration in PBS overnight at 4°C. A standard ELISA protocol was followed using detection of bound phage with anti-M13-HRP conjugate. ELISA results demonstrated that VH single domains specifically recognised APS when displayed on the surface of the filamentous bacteriophage.

Figure 10 shows the ELISA of soluble V_H2sd and V_H4sd. The same results are obtained as with the phage ELISA showing in figure 9, indicating that these single domains are also able to recognise APS or soluble fragments.

Figure 11 shows the selection of single V_H domain antibodies directed against APS and single V_K domain antibodies directed against β-gal from a repertoire of single antibody domains. Soluble single domain ELISA was performed as soluble scFv ELISA described in example 1 and bound V_K and V_H single

domains were detected with Protein L-HRP and Protein A-HRP respectively. Five VH single domains V_HA10sd, V_HA1sd, V_HA5sd, V_HC5sd and V_HC11sd selected from library 5 were found to bind APS and one V_K single domain V_KE5SD selected from library 6 was found to bind β-gal. None of the clones cross-reacted with BSA.

Figure 12 shows the characterisation of dual specific scFv antibodies V_KE5/V_H2 and V_KE5/V_H4 directed against APS and β-gal. Soluble scFv ELISA was performed as described in example 1 and the bound scFvs were detected with Protein L-HRP. Both V_KE5/V_H2 and V_KE5/V_H4 clones were found to be dual specific. No cross reactivity with BSA was detected.

Figure 13 shows the construction of V_K vector and V_KG3 vector. V_KG was pc amplified from an individual clone, A4 selected from a Fab library using BK BACKNOT as a 5' back primer and CKSACFORFL as a 3' (forward) primer. 30 cycles of PCR amplification was performed except that *Pfu* polymerase was used in enzyme. PCR product was digested with Not*I*/*EcoRI* and ligated into a Not*I*/*EcoRI* digested vector pHEN14V_K to create a C_K vector.

Figure 14 shows the C_K vector referred to in figure 13.

Figure 15 shows a Ck/gIII phagemid. Gene III was PCR amplified from a pIT2 vector using G3BACKSAC as a 5' (back) primer and LMB2 as a 3' (forward) primer. 30 cycles of PCR amplification were performed as described herein. PCR product was digested with SAC*I*/Eco*RI* and ligated into a Sac*I*/Eco*RI* digested C_K vector.

Figure 16 shows a C_H vector. C_H gene was PCR amplified from an individual clone A4 selected from a Fab library using CHBACKNOT as a 5' (back) primer and CHSACFOR as a 3' (forward) primer. 30 cycles of PCR amplification were performed as described herein. PCR product was

digested with a NotI/Bg1II and ligated into a NotI/BglII digested vector PACYC4V_H to create a C_H vector.

Figure 17 shows the C_H vector referred to in Figure 16.

Figure 18 shows an ELISA of V_KE5/V_H2 Fab.

Figure 19 shows competition ELISAs with V_KE5/V_H2 ScFv and V_KE5/V_H2 Fab.

Detailed Description of the invention

Definitions

Complementary Two immunoglobulin domains are "complementary" where they belong to or are derived from families of structures which form cognate pairs or groups. For example, a V_H domain and a V_L domain of an antibody are complementary; two V_H domains are not complementary, and two V_L domains are not complementary. Complementary domains may be found in other members of the immunoglobulin superfamily, such as the V_α and V_β (or γ and δ) domains of the T-cell receptor.

Immunoglobulin This refers to a family of polypeptides which retain the immunoglobulin fold characteristic of antibody molecules, which contains two β sheets and, usually, a conserved disulphide bond. Members of the immunoglobulin superfamily are involved in many aspects of cellular and non-cellular interactions *in vivo*, including widespread roles in the immune system (for example, antibodies, T-cell receptor molecules and the like), involvement in cell adhesion (for example the ICAM molecules) and intracellular signalling (for example, receptor molecules, such as the PDGF receptor). The present invention is applicable to all immunoglobulin superfamily molecules which display possess complementary domains. Preferably, the present invention relates to antibodies.

Combining Complementary variable domains according to the invention are combined to form a group of complementary domains; for example, V_L domains are combined with V_H domains. Domains may be combined in a number of ways, involving linkage of the domains by covalent or non-covalent means.

Domain A domain is a folded protein structure which retains its tertiary structure independently of the rest of the protein. Generally, domains are responsible for discrete functional properties of proteins, and in many cases may be added, removed or transferred to other proteins without loss of function. By single antibody variable domain we mean a folded polypeptide domain comprising sequences characteristic of antibody variable domains. It therefore includes antibody variable domains, for example in which one or more loops have been replaced by sequences which are not characteristic of antibody variable domains, or antibody variable domains which have been truncated or comprise N- or C-terminal extensions.

Repertoire A collection of diverse variants, for example polypeptide variants which differ in their primary sequence. A library used in the present invention will encompass a repertoire of polypeptides comprising at least 1000 members.

Library The term library refers to a mixture of heterogeneous polypeptides or nucleic acids. The library is composed of members, which have a single polypeptide or nucleic acid sequence. To this extent, *library* is synonymous with *repertoire*. Sequence differences between library members are responsible for the diversity present in the library. The library may take the form of a simple mixture of polypeptides or nucleic acids, or may be in the form organisms or cells, for example bacteria, viruses, animal or plant cells and the like, transformed with a library of nucleic acids. Preferably, each individual organism or cell contains only one or a limited number of library members. Advantageously, the nucleic acids are incorporated into expression vectors, in order to allow expression of the polypeptides encoded by the nucleic acids. In a preferred aspect, therefore, a library may take the form of a population of host organisms, each organism containing one or more copies of an expression vector containing a single member of the library in nucleic acid form which can be expressed to produce its corresponding polypeptide member. Thus, the population of host organisms has the potential to encode a large repertoire of genetically diverse polypeptide variants.

Antibody An antibody (for example IgG, IgM, IgA, IgD or IgE) or fragment (such as a FAb, $F(Ab')_2$, Fv, disulphide linked Fv, scFv, diabody) whether derived from any

species naturally producing an antibody, or created by recombinant DNA technology; whether isolated from serum, B-cells, hybridomas, transfectomas, yeast or bacteria).

Dual-specific ligand A ligand comprising a first immunoglobulin single variable domain and a second immunoglobulin single variable domain as herein defined, wherein the variable regions are capable of binding to two different antigens.

Antigen A ligand that binds to a small fraction of the members of a repertoire according to the present invention. It may be a polypeptide, protein, nucleic acid or other molecule. Generally, the immunoglobulin ligands according to the invention are selected for target specificity against a particular antigen. In the case of antibodies and fragments thereof, the antibody binding site defined by the variable loops (L1, L2, L3 and H1, H2, H3) is capable of binding to the antigen.

Specific generic ligand A ligand that binds to all members of a repertoire. Generally, not bound through the antigen binding site as defined above.

Selecting Derived by screening, or derived by a Darwinian selection process, in which binding interactions are made between a domain and the antigen or between an antibody and an antigen. Thus a first variable domain may be selected for binding to an antigen in the presence or in the absence of a complementary variable domain.

Universal framework A single antibody framework sequence corresponding to the regions of an antibody conserved in sequence as defined by Kabat ("Sequences of Proteins of Immunological Interest", US Department of Health and Human Services) or structure as defined by Chothia and Lesk, (1987) J. Mol. Biol. 196:910-917. The invention provides for the use of a single framework, or a set of such frameworks, which has been found to permit the derivation of virtually any binding specificity though variation in the hypervariable regions alone.

Detailed description of the invention

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridisation techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. which are incorporated herein by reference) and chemical methods.

Dual specific ligands according to the invention may be prepared according to previously established techniques, used in the field of antibody engineering, for the preparation of scFv, "phage" antibodies and other engineered antibody molecules. Techniques for the preparation of antibodies, and in particular bispecific antibodies, are for example described in the following reviews and the references cited therein: Winter & Milstein, (1991) Nature 349:293-299; Plueckthun (1992) Immunological Reviews 130:151-188; Wright *et al.*, (1992) Crti. Rev. Immunol.12:125-168; Holliger, P. & Winter, G. (1993) Curr. Op. Biotechn. 4, 446-449; Carter, *et al.* (1995) J. Hematother. 4, 463-470; Chester, K.A. & Hawkins, R.E. (1995) Trends Biotechn. 13, 294-300; Hoogenboom, H.R. (1997) Nature Biotechnol. 15, 125-126; Fearon, D. (1997) Nature Biotechnol. 15, 618-619; Plückthun, A. & Pack, P. (1997) Immunotechnology 3, 83-105; Carter, P. & Merchant, A.M. (1997) Curr. Opin. Biotechnol. 8, 449-454; Holliger, P. & Winter, G. (1997) Cancer Immunol. Immunother. 45,128-130.

The invention provides for the selection of complementary variable domains against two different antigens, and subsequent combination of the variable domains.

The techniques employed for selection of the variable domains employ libraries and selection procedures which are known in the art. Natural libraries (Marks *et al.* (1991) *J. Mol. Biol.*, 222: 581; Vaughan *et al.* (1996) *Nature Biotech.*, 14: 309) which use rearranged V genes harvested from human B cells are well known to those skilled in the art. Synthetic libraries (Hoogenboom & Winter (1992) *J. Mol. Biol.*, 227: 381; Barbas *et*

al. (1992) *Proc. Natl. Acad. Sci. USA*, 89: 4457; Nissim *et al.* (1994) *EMBO J.*, 13: 692; Griffiths *et al.* (1994) *EMBO J.*, 13: 3245; De Kruif *et al.* (1995) *J. Mol. Biol.*, 248: 97) are prepared by cloning immunoglobulin V genes, usually using PCR. Errors in the PCR process can lead to a high degree of randomisation. V_H and/or V_L libraries may be selected against target antigens separately, in which case single domain binding is directly selected for, or together.

A preferred method for making a dual specific ligand according to the present invention comprises using a selection system in which a repertoire of variable domains is selected for binding to a first antigen and a repertoire of variable domains is selected for binding to a second antigen. The selected variable first and second variable domains are then combined and the dual-specific selected for binding to both first and second antigen.

A. Library vector systems

A variety of selection systems are known in the art which are suitable for use in the present invention. Examples of such systems are described below.

Bacteriophage lambda expression systems may be screened directly as bacteriophage plaques or as colonies of lysogens, both as previously described (Huse *et al.* (1989) *Science*, 246: 1275; Caton and Koprowski (1990) *Proc. Natl. Acad. Sci. U.S.A.*, 87; Mullinax *et al.* (1990) *Proc. Natl. Acad. Sci. U.S.A.*, 87: 8095; Persson *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.*, 88: 2432) and are of use in the invention. Whilst such expression systems can be used to screening up to 10^6 different members of a library, they are not really suited to screening of larger numbers (greater than 10^6 members).

Of particular use in the construction of libraries are selection display systems, which enable a nucleic acid to be linked to the polypeptide it expresses. As used herein, a selection display system is a system that permits the selection, by suitable display means, of the individual members of the library by binding the generic and/or target ligands.

Selection protocols for isolating desired members of large libraries are known in the art, as typified by phage display techniques. Such systems, in which diverse peptide

sequences are displayed on the surface of filamentous bacteriophage (Scott and Smith (1990) *Science*, **249**: 386), have proven useful for creating libraries of antibody fragments (and the nucleotide sequences that encoding them) for the *in vitro* selection and amplification of specific antibody fragments that bind a target antigen (McCafferty et al., WO 92/01047). The nucleotide sequences encoding the V_H and V_L regions are linked to gene fragments which encode leader signals that direct them to the periplasmic space of *E. coli* and as a result the resultant antibody fragments are displayed on the surface of the bacteriophage, typically as fusions to bacteriophage coat proteins (e.g., pIII or pVIII). Alternatively, antibody fragments are displayed externally on lambda phage capsids (phagebodies). An advantage of phage-based display systems is that, because they are biological systems, selected library members can be amplified simply by growing the phage containing the selected library member in bacterial cells. Furthermore, since the nucleotide sequence that encode the polypeptide library member is contained on a phage or phagemid vector, sequencing, expression and subsequent genetic manipulation is relatively straightforward.

Methods for the construction of bacteriophage antibody display libraries and lambda phage expression libraries are well known in the art (McCafferty *et al.* (1990) *Nature*, **348**: 552; Kang *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.*, **88**: 4363; Clackson *et al.* (1991) *Nature*, **352**: 624; Lowman *et al.* (1991) *Biochemistry*, **30**: 10832; Burton *et al.* (1991) *Proc. Natl. Acad. Sci U.S.A.*, **88**: 10134; Hoogenboom *et al.* (1991) Nucleic Acids Res., **19**: 4133; Chang *et al.* (1991) *J. Immunol.*, **147**: 3610; Breitling *et al.* (1991) *Gene*, **104**: 147; Marks *et al.* (1991) supra; Barbas *et al.* (1992) supra; Hawkins and Winter (1992) *J. Immunol.*, **22**: 867; Marks *et al.*, 1992, *J. Biol. Chem.*, **267**: 16007; Lerner *et al.* (1992) *Science*, **258**: 1313, incorporated herein by reference).

One particularly advantageous approach has been the use of scFv phage-libraries (Huston *et al.*, 1988, Proc. Natl. Acad. Sci U.S.A., 85: 5879-5883; Chaudhary *et al.* (1990) Proc. Natl. Acad. Sci U.S.A., 87: 1066-1070; McCafferty *et al.* (1990) supra; Clackson *et al.* (1991) *Nature*, **352**: 624; Marks *et al.* (1991) *J. Mol. Biol.*, **222**: 581; Chiswell *et al.* (1992) Trends Biotech., **10**: 80; Marks *et al.* (1992) *J. Biol. Chem.*, **267**). Various embodiments of scFv libraries displayed on bacteriophage coat proteins have been described. Refinements of phage display approaches are also known, for example as

described in WO96/06213 and WO92/01047 (Medical Research Council *et al.*) and WO97/08320 (Morphosys), which are incorporated herein by reference.

Other systems for generating libraries of polypeptides involve the use of cell-free enzymatic machinery for the *in vitro* synthesis of the library members. In one method, RNA molecules are selected by alternate rounds of selection against a target ligand and PCR amplification (Tuerk and Gold (1990) *Science*, 249: 505; Ellington and Szostak (1990) *Nature*, 346: 818). A similar technique may be used to identify DNA sequences which bind a predetermined human transcription factor (Thiesen and Bach (1990) *Nucleic Acids Res.*, 18: 3203; Beaudry and Joyce (1992) *Science*, 257: 635; WO92/05258 and WO92/14843). In a similar way, *in vitro* translation can be used to synthesise polypeptides as a method for generating large libraries. These methods which generally comprise stabilised polysome complexes, are described further in WO88/08453, WO90/05785, WO90/07003, WO91/02076, WO91/05058, and WO92/02536. Alternative display systems which are not phage-based, such as those disclosed in WO95/22625 and WO95/11922 (Affymax) use the polysomes to display polypeptides for selection.

A still further category of techniques involves the selection of repertoires in artificial compartments, which allow the linkage of a gene with its gene product. For example, a selection system in which nucleic acids encoding desirable gene products may be selected in microcapsules formed by water-in-oil emulsions is described in WO99/02671, WO00/40712 and Tawfik & Griffiths (1998) *Nature Biotechnol* 16(7), 652-6. Genetic elements encoding a gene product having a desired activity are compartmentalised into microcapsules and then transcribed and/or translated to produce their respective gene products (RNA or protein) within the microcapsules. Genetic elements which produce gene product having desired activity are subsequently sorted. This approach selects gene products of interest by detecting the desired activity by a variety of means.

B. Library Construction.

Libraries intended for selection, may be constructed using techniques known in the art, for example as set forth above, or may be purchased from commercial sources. Libraries

which are useful in the present invention are described, for example, in WO99/20749. Once a vector system is chosen and one or more nucleic acid sequences encoding polypeptides of interest are cloned into the library vector, one may generate diversity within the cloned molecules by undertaking mutagenesis prior to expression; alternatively, the encoded proteins may be expressed and selected, as described above, before mutagenesis and additional rounds of selection are performed. Mutagenesis of nucleic acid sequences encoding structurally optimised polypeptides is carried out by standard molecular methods. Of particular use is the polymerase chain reaction, or PCR, (Mullis and Faloona (1987) *Methods Enzymol.*, 155: 335, herein incorporated by reference). PCR, which uses multiple cycles of DNA replication catalysed by a thermostable, DNA-dependent DNA polymerase to amplify the target sequence of interest, is well known in the art. The construction of various antibody libraries has been discussed in Winter *et al.* (1994) Ann. Rev. Immunology 12, 433-55, and references cited therein.

PCR is performed using template DNA (at least 1fg; more usefully, 1-1000 ng) and at least 25 pmol of oligonucleotide primers; it may be advantageous to use a larger amount of primer when the primer pool is heavily heterogeneous, as each sequence is represented by only a small fraction of the molecules of the pool, and amounts become limiting in the later amplification cycles. A typical reaction mixture includes: 2µl of DNA, 25 pmol of oligonucleotide primer, 2.5 µl of 10X PCR buffer 1 (Perkin-Elmer, Foster City, CA), 0.4 µl of 1.25 µM dNTP, 0.15 µl (or 2.5 units) of Taq DNA polymerase (Perkin Elmer, Foster City, CA) and deionized water to a total volume of 25 µl. Mineral oil is overlaid and the PCR is performed using a programmable thermal cycler. The length and temperature of each step of a PCR cycle, as well as the number of cycles, is adjusted in accordance to the stringency requirements in effect. Annealing temperature and timing are determined both by the efficiency with which a primer is expected to anneal to a template and the degree of mismatch that is to be tolerated; obviously, when nucleic acid molecules are simultaneously amplified and mutagenized, mismatch is required, at least in the first round of synthesis. The ability to optimise the stringency of primer annealing conditions is well within the knowledge of one of moderate skill in the art. An annealing temperature of between 30 C and 72 °C is used. Initial denaturation of the template molecules normally occurs at between 92°C and 99°C for 4 minutes, followed by 20-40

cycles consisting of denaturation (94-99°C for 15 seconds to 1 minute), annealing (temperature determined as discussed above; 1-2 minutes), and extension (72°C for 1-5 minutes, depending on the length of the amplified product). Final extension is generally for 4 minutes at 72°C, and may be followed by an indefinite (0-24 hour) step at 4°C.

C. Combining complementary single domains

Domains according to the invention, once selected, may be combined by a variety of methods known in the art, including covalent and non-covalent methods.

Preferred methods include the use of polypeptide linkers, as described, for example, in connection with scFv molecules (Bird *et al.*, (1988) Science 242:423-426). Linkers are preferably flexible, allowing the two single domains to interact. The linkers used in diabodies, which are less flexible, may also be employed (Holliger *et al.*, (1993) PNAS (USA) 90:6444-6448).

Complementary variable domains may be combined using methods other than linkers. For example, the use of disulphide bridges, provided through naturally-occurring or engineered cysteine residues, may be exploited to stabilise V_H-V_L dimers (Reiter *et al.*, (1994) Protein Eng. 7:697-704) or by remodelling the interface between the variable domains to improve the "fit" and thus the stability of interaction (Ridgeway *et al.*, (1996) Protein Eng. 7:617-621; Zhu *et al.*, (1997) Protein Science 6:781-788).

Other techniques for joining or stabilising variable domains of immunoglobulins, and in particular antibody V_H and V_L domains, may be employed as appropriate.

In accordance with the present invention, it is envisaged that dual specific ligands may exist in "open" or "closed" conformations in solution. An "open" conformation is a conformation in which each of the immunoglobulin domains is present in a form unassociated with other domains; in other words, each domain is present as a single domain in solution. The "closed" configuration is that in which the two domains (for example V_H and V_L) are present in associated form, such as that of an associated V_H-V_L pair which forms an antibody binding site.

Open and closed dual specific ligands are likely to exist in a variety of equilibria under different circumstances. Binding of the ligand to a target is likely to shift the balance of the equilibrium towards the open configuration. Thus, the ligands according to the invention can exist in two conformations in solution, one of which (the open form) can bind two antigens independently, whilst the alternative conformation (the closed form) can only bind one antigen; antigens thus compete for binding to the ligand in this conformation.

Although the open form of the dual specific ligand may thus exist in equilibrium with the closed form solution, it is envisaged that the equilibrium will favour the closed form; moreover, the open form can be sequestered by target binding into a closed conformation. Preferably, therefore, the dual specific ligand of the invention is present in an equilibrium between two (open and closed) conformations.

Dual specific ligands according to the invention may be modified in order to favour an open or closed conformation. For example, stabilisation of V_H-V_L interactions with disulphide bonds stabilises the closed conformation. Moreover, linkers used to join the domains may be constructed such that the open from is favoured; for example, the linkers may sterically hinder the association of the domains, such as by incorporation of large amino acid residues in opportune locations, or the designing of a suitable rigid structure which will keep the domains physically spaced apart.

D. Characterisation of the dual-specific ligand.

The binding of the dual-specific ligand to its specific antigens can be tested by methods which will be familiar to those skilled in the art and include ELISA. In a preferred embodiment of the invention binding is tested using monoclonal phage ELISA.

Phage ELISA may be performed according to any suitable procedure: an exemplary protocol is set forth below.

Populations of phage produced at each round of selection can be screened for binding by ELISA to the selected antigen, to identify "polyclonal" phage antibodies. Phage from single infected bacterial colonies from these populations can then be screened by ELISA to identify "monoclonal" phage antibodies. It is also desirable to screen soluble antibody fragments for binding to antigen, and this can also be undertaken by ELISA using reagents, for example, against a C- or N-terminal tag (see for example Winter *et al.* (1994) Ann. Rev. Immunology 12, 433-55 and references cited therein.

The diversity of the selected phage monoclonal antibodies may also be assessed by gel electrophoresis of PCR products (Marks et al. 1991, *supra*; Nissim et al. 1994 *supra*), probing (Tomlinson *et al.*, 1992) J. Mol. Biol. 227, 776) or by sequencing of the vector DNA.

E. Structure of 'Dual-specific ligands'.

As described above, an antibody is herein defined as an antibody (for example IgG, IgM, IgA, IgA, IgE) or fragment (Fab, Fv, disulphide linked Fv, scFv, diabody) which comprises at least one heavy and a light chain variable domain which are complementary to one another and thus can associate with one another to form a VH/VL pair. It may be derived from any species naturally producing an antibody, or created by recombinant DNA technology; whether isolated from serum, B-cells, hybridomas, transfectomas, yeast or bacteria).

In a preferred embodiment of the invention the dual-specific ligand comprises at least one single heavy chain variable domain of an antibody and one single light chain variable domain of an antibody such that the two regions are capable of associating to form a complementary VH/VL pair.

The first and the second variable domains of such a ligand may be on the same polypeptide chain. Alternatively they may be on separate polypeptide chains, In the case that they are on the same polypeptide chain they may be linked by a flexible linker, which is preferentially a peptide sequence, as described above.

The first and second variable domains may be covalently or non-covalently associated. In the case that they are covalently associated, the covalent bonds may be disulphide bonds.

In the case that the variable domains are selected from V-gene repertoires selected for instance using phage display technology as herein described, then these variable domains comprise a universal framework region, such that is they may be recognised by a specific generic ligand as herein defined. The use of universal frameworks, generic ligands and the like is described in WO99/20749.

Where V-gene repertoires are used variation in polypeptide sequence is preferably located within the structural loops of the variable domains. The polypeptide sequences of either variable domain may be altered by DNA shuffling or by mutation in order to enhance the interaction of each variable domain with its complementary pair.

In a preferred embodiment of the invention the 'dual-specific ligand' is a single chain Fv fragment. In an alternative embodiment of the invention, the 'dual-specific ligand' comprises a Fab region of an antibody

In a further aspect, the present invention provides nucleic acid encoding at least a 'dual-specific ligand' as herein defined.

One skilled in the art will appreciate that both antigens may bind simultaneously to the same antibody molecule. Alternatively, they may compete for binding to the same antibody molecule.

The variable regions may be derived from antibodies directed against target antigens. Alternatively they may be derived from a repertoire of single antibody domains such as those expressed on the surface of filamentous bacteriophage. Selection may be performed as described below.

In general, the nucleic acid molecules and vector constructs required for the performance of the present invention may be constructed and manipulated as set forth in standard

laboratory manuals, such as Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, USA.

The manipulation of nucleic acids in the present invention is typically carried out in recombinant vectors.

Thus in a further aspect, the present invention provides a vector comprising nucleic acid encoding at least a 'dual-specific ligand' as herein defined.

As used herein, vector refers to a discrete element that is used to introduce heterologous DNA into cells for the expression and/or replication thereof. Methods by which to select or construct and, subsequently, use such vectors are well known to one of moderate skill in the art. Numerous vectors are publicly available, including bacterial plasmids, bacteriophage, artificial chromosomes and episomal vectors. Such vectors may be used for simple cloning and mutagenesis; alternatively gene expression vector is employed. A vector of use according to the invention may be selected to accommodate a polypeptide coding sequence of a desired size, typically from 0.25 kilobase (kb) to 40 kb or more in length A suitable host cell is transformed with the vector after *in vitro* cloning manipulations. Each vector contains various functional components, which generally include a cloning (or "polylinker") site, an origin of replication and at least one selectable marker gene. If given vector is an expression vector, it additionally possesses one or more of the following: enhancer element, promoter, transcription termination and signal sequences, each positioned in the vicinity of the cloning site, such that they are operatively linked to the gene encoding a polypeptide repertoire member according to the invention.

Both cloning and expression vectors generally contain nucleic acid sequences that enable the vector to replicate in one or more selected host cells. Typically in cloning vectors, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 micron plasmid origin is suitable for yeast, and various viral origins (e.g.

SV 40, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication is not needed for mammalian expression vectors unless these are used in mammalian cells able to replicate high levels of DNA, such as COS cells.

Advantageously, a cloning or expression vector may contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will therefore not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g. ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available in the growth media.

Since the replication of vectors according to the present invention is most conveniently performed in *E. coli*, an *E. coli*-selectable marker, for example, the β-lactamase gene that confers resistance to the antibiotic ampicillin, is of use. These can be obtained from *E. coli* plasmids, such as pBR322 or a pUC plasmid such as pUC18 or pUC19.

Expression vectors usually contain a promoter that is recognised by the host organism and is operably linked to the coding sequence of interest. Such a promoter may be inducible or constitutive. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

Promoters suitable for use with prokaryotic hosts include, for example, the β-lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (trp) promoter system and hybrid promoters such as the tac promoter. Promoters for use in bacterial systems will also generally contain a Shine-Delgarno sequence operably linked to the coding sequence.

The preferred vectors are expression vectors that enables the expression of a nucleotide sequence corresponding to a polypeptide library member. Thus, selection with the first and/or second antigen can be performed by separate propagation and expression of a single clone expressing the polypeptide library member or by use of any selection display system. As described above, the preferred selection display system is bacteriophage display: Thus, phage or phagemid vectors may be used. The preferred vectors are phagemid vectors which have an *E. coli.* origin of replication (for double stranded replication) and also a phage origin of replication (for production of single-stranded DNA). The manipulation and expression of such vectors is well known in the art (Hoogenboom and Winter (1992) supra; Nissim *et al.* (1994) supra). Briefly, the vector contains a β-lactamase gene to confer selectivity on the phagemid and a lac promoter upstream of a expression cassette that consists (N to C terminal) of a pelB leader sequence (which directs the expressed polypeptide to the periplasmic space), a multiple cloning site (for cloning the nucleotide version of the library member), optionally, one or more peptide tag (for detection), optionally, one or more TAG stop codon and the phage protein pIII. Thus, using various suppressor and non-suppressor strains of *E. coli* and with the addition of glucose, iso-propyl thio-β-D-galactoside (IPTG) or a helper phage, such as VCS M13, the vector is able to replicate as a plasmid with no expression, produce large quantities of the polypeptide library member only or produce phage, some of which contain at least one copy of the polypeptide-pIII fusion on their surface.

Construction of vectors according to the invention employs conventional ligation techniques. Isolated vectors or DNA fragments are cleaved, tailored, and religated in the form desired to generate the required vector. If desired, analysis to confirm that the correct sequences are present in the constructed vector can be performed in a known fashion. Suitable methods for constructing expression vectors, preparing *in vitro* transcripts, introducing DNA into host cells, and performing analyses for assessing expression and function are known to those skilled in the art. The presence of a gene sequence in a sample is detected, or its amplification and/or expression quantified by conventional methods, such as Southern or Northern analysis, Western blotting, dot blotting of DNA, RNA or protein, *in situ* hybridisation, immunocytochemistry or sequence analysis of nucleic acid or protein molecules. Those skilled in the art will readily envisage how these methods may be modified, if desired.

F: Use of dual-specific ligands selected according to the invention

Dual-specific ligands selected according to the method of the present invention may be employed in in vivo therapeutic and prophylactic applications, in vitro and in vivo diagnostic applications, in vitro assay and reagent applications, and the like. For example antibody molecules may be used in antibody based assay techniques, such as ELISA techniques, according to methods known to those skilled in the art.

As alluded to above, the molecules selected according to the invention are of use in diagnostic, prophylactic and therapeutic procedures. Dual specific antibodies selected according to the invention are of use diagnostically in Western analysis and *in situ* protein detection by standard immunohistochemical procedures; for use in these applications, the antibodies of a selected repertoire may be labelled in accordance with techniques known to the art. In addition, such antibody polypeptides may be used preparatively in affinity chromatography procedures, when complexed to a chromatographic support, such as a resin. All such techniques are well known to one of skill in the art.

Therapeutic and prophylactic uses of dual-specific ligands prepared according to the invention involve the administration of ligands selected according to the invention to a recipient mammal, such as a human. Dual-specificity can allow antibodies to bind to multimeric antigen with great avidity. Dual-specific antibodies can allow the cross-linking of two antigens, for example in recruiting cytotoxic T-cells to mediate the killing of tumour cell lines.

Substantially pure antibodies or binding proteins thereof of at least 90 to 95% homogeneity are preferred for administration to a mammal, and 98 to 99% or more homogeneity is most preferred for pharmaceutical uses, especially when the mammal is a human. Once purified, partially or to homogeneity as desired, the selected polypeptides may be used diagnostically or therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings and the like (Lefkovite and Pernis, (1979 and 1981) Immunological Methods, Volumes I and II, Academic Press, NY).

The selected antibodies or binding proteins thereof of the present invention will typically find use in preventing, suppressing or treating inflammatory states, allergic hypersensitivity, cancer, bacterial or viral infection, and autoimmune disorders (which include, but are not limited to, Type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease and myasthenia gravis).

In the instant application, the term "prevention" involves administration of the protective composition prior to the induction of the disease. "Suppression" refers to administration of the composition after an inductive event, but prior to the clinical appearance of the disease. "Treatment" involves administration of the protective composition after disease symptoms become manifest.

Animal model systems which can be used to screen the effectiveness of the antibodies or binding proteins thereof in protecting against or treating the disease are available. Methods for the testing of systemic lupus erythematosus (SLE) in susceptible mice are known in the art (Knight *et al.* (1978) *J. Exp. Med.*, **147**: 1653; Reinersten *et al.* (1978) *New Eng. J. Med.*, **299**: 515). Myasthenia Gravis (MG) is tested in SJL/J female mice by inducing the disease with soluble AchR protein from another species (Lindstrom *et al.* (1988) *Adv. Immunol.*, **42**: 233). Arthritis is induced in a susceptible strain of mice by injection of Type II collagen (Stuart *et al.* (1984) *Ann. Rev. Immunol.*, **42**: 233). A model by which adjuvant arthritis is induced in susceptible rats by injection of mycobacterial heat shock protein has been described (Van Eden *et al.* (1988) *Nature*, **331**: 171). Thyroiditis is induced in mice by administration of thyroglobulin as described (Maron *et al.* (1980) *J. Exp. Med.*, **152**: 1115). Insulin dependent diabetes mellitus (IDDM) occurs naturally or can be induced in certain strains of mice such as those described by Kanasawa *et al.* (1984) *Diabetologia*, **27**: 113. EAE in mouse and rat serves as a model for MS in human. In this model, the demyelinating disease is induced by administration of myelin basic protein (see Paterson (1986) *Textbook of Immunopathology*, Mischer *et al.*, eds., Grune and Stratton, New York, pp. 179-213; McFarlin *et al.* (1973) *Science*, **179**: 478: and Satoh *et al.* (1987) *J. Immunol.*, **138**: 179).

Generally, the present selected antibodies will be utilised in purified form together with pharmacologically appropriate carriers. Typically, these carriers include aqueous or alcoholic/aqueous solutions, emulsions or suspensions, any including saline and/or buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride and lactated Ringer's. Suitable physiologically-acceptable adjuvants, if necessary to keep a polypeptide complex in suspension, may be chosen from thickeners such as carboxymethylcellulose, polyvinylpyrrolidone, gelatin and alginates.

Intravenous vehicles include fluid and nutrient replenishers and electrolyte replenishers, such as those based on Ringer's dextrose. Preservatives and other additives, such as antimicrobials, antioxidants, chelating agents and inert gases, may also be present (Mack (1982) *Remington's Pharmaceutical Sciences*, 16th Edition).

The selected polypeptides of the present invention may be used as separately administered compositions or in conjunction with other agents. These can include various immunotherapeutic drugs, such as cylcosporine, methotrexate, adriamycin or cisplatinum, and immunotoxins. Pharmaceutical compositions can include "cocktails" of various cytotoxic or other agents in conjunction with the selected antibodies, receptors or binding proteins thereof of the present invention, or even combinations of selected polypeptides according to the present invention having different specificities, such as polypeptides selected using different target ligands, whether or not they are pooled prior to administration.

The route of administration of pharmaceutical compositions according to the invention may be any of those commonly known to those of ordinary skill in the art. For therapy, including without limitation immunotherapy, the selected antibodies, receptors or binding proteins thereof of the invention can be administered to any patient in accordance with standard techniques. The administration can be by any appropriate mode, including parenterally, intravenously, intramuscularly, intraperitoneally, transdermally, *via* the pulmonary route, or also, appropriately, by direct infusion with a catheter. The dosage and frequency of administration will depend on the age, sex and condition of the patient, concurrent administration of other drugs, counterindications and other parameters to be taken into account by the clinician.

The selected polypeptides of this invention can be lyophilised for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immunoglobulins and art-known lyophilisation and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilisation and reconstitution can lead to varying degrees of antibody activity loss (e.g. with conventional immunoglobulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted upward to compensate.

The compositions containing the present selected polypeptides or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In certain therapeutic applications, an adequate amount to accomplish at least partial inhibition, suppression, modulation, killing, or some other measurable parameter, of a population of selected cells is defined as a "therapeutically-effective dose". Amounts needed to achieve this dosage will depend upon the severity of the disease and the general state of the patient's own immune system, but generally range from 0.005 to 5.0 mg of selected antibody, receptor (e.g. a T-cell receptor) or binding protein thereof *per* kilogram of body weight, with doses of 0.05 to 2.0 mg/kg/dose being more commonly used. For prophylactic applications, compositions containing the present selected polypeptides or cocktails thereof may also be administered in similar or slightly lower dosages.

A composition containing a selected polypeptide according to the present invention may be utilised in prophylactic and therapeutic settings to aid in the alteration, inactivation, killing or removal of a select target cell population in a mammal. In addition, the selected repertoires of polypeptides described herein may be used extracorporeally or *in vitro* selectively to kill, deplete or otherwise effectively remove a target cell population from a heterogeneous collection of cells. Blood from a mammal may be combined extracorporeally with the selected antibodies, cell-surface receptors or binding proteins thereof whereby the undesired cells are killed or otherwise removed from the blood for return to the mammal in accordance with standard techniques.

The invention is further described, for the purposes of illustration only, in the following examples.

Example 1. Selection of a dual specific scFv antibody (K8) directed against human serum albumin (HSA) and β-galactosidase (β -gal)

This example explains a method for making a dual specific antibody directed against β-gal and HSA in which a repertoire of V_K variable domains linked to a germline (dummy) V_H domain is selected for binding to β-gal and a repertoire of V_H variable domains linked to a germline (dummy) V_K domain is selected for binding to HSA. The selected variable V_H HSA and V_K β-gal domains are then combined and the antibodies selected for binding to β-gal and HSA.

Four human phage antibody libraries were used in this experiment.

Library 1	Germline V_K/DVT V_H	8.46×10^7
Library 2	Germline V_K/NNK V_H	9.64×10^7
Library 3	Germline V_H/DVT V_K	1.47×10^8
Library 4	Germline V_H/NNK V_K	1.45×10^8

All libraries are based on a single human framework for V_H (V3-23/DP47 and J_H4b) and V_K (O12/O2/DPK9 and J_K1) with side chain diversity incorporated in complementarity determining regions (CDR2 and CDR3).

Library 1 and Library 2 contain a dummy V_K sequence, whereas the sequence of V_H is diversified at positions H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97 and H98 (DVT or NNK encoded, respectively) (Figure 1). Library 3 and Library 4 contain a dummy V_H sequence, whereas the sequence of V_K is diversified at positions L50, L53, L91, L92, L93, L94 and L96 (DVT or NNK encoded, respectively) (Figure 1). The libraries are in phagemid pIT2/ScFv format (Figure 2) and have been preselected for binding to generic ligands, Protein A and Protein L, so that the majority of clones in the

unselected libraries are functional. The sizes of the libraries shown above correspond to the sizes after preselection. Library 1 and Library 2 were mixed prior to selections on antigen to yield a single V_H/dummy V_K library and Library 3 and Library 4 were mixed to form a single V_K/dummy V_H library.

Three rounds of selections were performed on β-gal using V_K/dummy V_H library and three rounds of selections were performed on HSA using V_H/dummy V_K library. In the case of β-gal the phage titres went up from 1.1×10^6 in the first round to 2.0×10^8 in the third round. In the case of HSA the phage titres went up from 2×10^4 in the first round to 1.4×10^9 in the third round. The selections were performed as described by Griffith *et al.*, (1993), except that KM13 helper phage (which contains a pIII protein with a protease cleavage site between the D2 and D3 domains) was used and phage were eluted with 1 mg/ml trypsin in PBS. The addition of trypsin cleaves the pIII proteins derived from the helper phage (but not those from the phagemid) and elutes bound scFv-phage fusions by cleavage in the c-myc tag (Figure 2), thereby providing a further enrichment for phages expressing functional scFvs and a corresponding reduction in background (Kristensen & Winter, 1998). Selections were performed using immunotubes coated with either HSA or β-gal at 100μg/ml concentration.

To check for binding, 24 colonies from the third round of each selection were screened by monoclonal phage ELISA. Phage particles were produced as described by Harrison *et al.*, (1996). 96-well ELISA plates were coated with 100μl of HSA or β-gal at 10μg/ml concentration in PBS overnight at 4°C. A standard ELISA protocol was followed (Hoogenboom *et al.*, 1991) using detection of bound phage with anti-M13-HRP conjugate. A selection of clones gave ELISA signals of greater than 1.0 with 50μl supernatant (data not shown).

Next, DNA preps were made from V_H/dummy V_K library selected on HSA and from V_K/dummy V_H library selected on β-gal using the QIAprep Spin Miniprep kit (Qiagen). To access most of the diversity, DNA preps were made from each of the three rounds of selections and then pulled together for each of the antigens. DNA preps were then

digested with *SalI*/*NotI* overnight at 37°C. Following gel purification of the fragments, V_K chains from the V_K/dummy V_H library selected on β-gal were ligated in place of a dummy V_K chain of the V_H/dummy V_K library selected on HSA creating a library of 3.3 x 10^9 clones.

This library was then either selected on HSA (first round) and β-gal (second round), HSA/β-gal selection, or on β-gal (first round) and HSA (second round), β-gal/HSA selection. Selections were performed as described above. In each case after the second round 48 clones were tested for binding to HSA and β-gal by the monoclonal phage ELISA (as described above) and by ELISA of the soluble scFv fragments. Soluble antibody fragments were produced as described by Harrison *et al.*, (1996), and standard ELISA protocol was followed (Hoogenboom *et al.*, 1991), except that 2% Tween/PBS was used as a blocking buffer and bound scFvs were detected with Protein L-HRP. Three clones (E4, E5 and E8) from the HSA/β-gal selection and two clones (K8 and K10) from the β-gal/HSA selection were able to bind both antigens (data not shown). scFvs from these clones were PCR amplified and sequenced as described by Ignatovich *et al.*, (1999) using the primers LMB3 and pHENseq (Table 1). Sequence analysis revealed that all clones were identical. Therefore, only one clone encoding a dual specific antibody (K8) was chosen for further work (Figure 3).

Example 2. Characterisation of the binding properties of the K8 antibody.

Firstly, the binding properties of the K8 antibody were characterised by the monoclonal phage ELISA. A 96-well plate was coated with 100μl of HSA and β-gal alongside with alkaline phosphatase (APS), bovine serum albumin (BSA), peanut agglutinin, lysozyme and cytochrome c (to check for cross-reactivity) at 10μg/ml concentration in PBS overnight at 4°C. The phagemid from K8 clone was rescued with KM13 as described by Harrison *et al.*, (1996) and the supernatant (50μl) containing phage assayed directly. A standard ELISA protocol was followed (Hoogenboom *et al.*, 1991) using detection of bound phage with anti-M13-HRP conjugate. The dual specific K8 antibody was found to bind to HSA and β-gal when displayed on the surface of the phage with absorbance

signals greater than 1.0 (Figure 4). Strong binding to BSA was also observed (Figure 4). Since HSA and BSA are 76% homologous on the amino acid level, it is not surprising that K8 antibody recognised both of these structurally related proteins. No cross-reactivity with other proteins was detected (Figure 4).

Secondly, the binding properties of the K8 antibody were tested in a soluble scFv ELISA. Production of the soluble scFv fragment was induced by IPTG as described by Harrison *et al.*, (1996). To determine the expression levels of K8 scFv, the soluble antibody fragments were purified from the supernatant of 50ml inductions using Protein A-Sepharose columns as described by Harlow & Lane (1988). OD_{280} was then measured and the protein concentration calculated as described by Sambrook *et al.*, (1989). K8 scFv was produced in supernatant at 19mg/l.

A soluble scFv ELISA was then performed using known concentrations of the K8 antibody fragment. A 96-well plate was coated with 100µl of HSA, BSA and β-gal at 10µg/ml and 100µl of Protein A at 1µg/ml concentration. 50µl of the serial dilutions of the K8 scFv was applied and the bound antibody fragments were detected with Protein L-HRP. ELISA results confirmed the dual specific nature of the K8 antibody (Figure 5).

To confirm that binding to β-gal is determined by the V_K domain and binding to HSA/BSA by the V_H domain of the K8 scFv antibody, the V_K domain was cut out from K8 scFv DNA by *SalI*/*NotI* digestion and ligated into a *SalI*/*NotI* digested pIT2 vector containing dummy V_H chain (Figures 1 and 2). Binding characteristics of the resulting clone K8V_K/dummy V_H were analysed by soluble scFv ELISA. Production of the soluble scFv fragments was induced by IPTG as described by Harrison *et al.*, (1996) and the supernatant (50µ) containing scFvs assayed directly. Soluble scFv ELISA was performed as described in Example 1 and the bound scFvs were detected with Protein L-HRP. The ELISA results revealed that this clone was still able to bind β-gal, whereas binding to BSA was abolished (Figure 6).

Example 3. Creation and characterisation of dual specific scFv antibodies ($K8V_K/V_H2$ and $K8V_K/V_H4$) directed against APS and β-gal and of a dual specific scFv antibody ($K8V_K/V_HC11$) directed against BCL10 protein and β-gal.

This example describes a method for making dual specific scFv antibodies ($K8V_K/V_H2$ and $K8V_K/V_H4$) directed against APS and β-gal and a dual specific scFv antibody ($K8V_K/V_HC11$) directed against BCL10 protein and β-gal, whereby a repertoire of V_H variable domains linked to a germline (dummy) V_K domain is first selected for binding to APS and BCL10 protein. The selected individual V_H domains (V_H2, V_H4 and V_HC11) are then combined with an individual β-gal binding V_K domain (from K8 scFv, Examples 1 and 2) and antibodies are tested for dual specificity.

A V_H/dummy V_K scFv library described in Example 1 was used to perform three rounds of selections on APS and two rounds of selections BCL10 protein. BCL10 protein is involved in the regulation of apoptosis and mutant forms of this protein are found in multiple tumour types, indicating that BCL10 may be commonly involved in the pathogenesis of human cancer (Willis *et al.*, 1999).

In the case of APS the phage titres went up from 2.8×10^5 in the first round to 8.0×10^8 in the third round. In the case of BCL10 the phage titres went up from 1.8×10^5 in the first round to 9.2×10^7 in the second round. The selections were performed as described in Example 1 using immunotubes coated with either APS or BCL10 at 100μg/ml concentration.

To check for binding, 24 colonies from the third round of APS selections and 48 colonies from the second round of the BCL10 selections were screened by soluble scFv ELISA. A 96-well plate was coated with 100μl of APS, BCL10, BSA, HSA and β-gal at 10μg/ml concentration in PBS overnight at 4°C. Production of the soluble scFv fragments was induced by IPTG as described by Harrison *et al.*, (1996) and the supernatant (50μl) containing scFvs assayed directly. Soluble scFv ELISA was performed as described in Example 1 and the bound scFvs were detected with Protein L-HRP. Two clones (V_H2

and V_H4) were found to bind APS and one clone (V_HC11) was specific for BCL10 (Figures 3, 7). No cross-reactivity with other proteins was observed.

To create dual specific antibodies each of these clones was digested with *SalI/NotI* to remove dummy V_K chains and a *SalI/NotI* fragment containing β-gal binding V_K domain from K8 scFv was ligated instead. The binding characteristics of the produced clones ($K8V_K/V_H2$, $K8V_K/V_H4$ and $K8V_K/V_HC11$) were tested in a soluble scFv ELISA as described above. All clones were found to be dual specific without any cross-reactivity with other proteins (Figure 8).

Example 4. Creation and characterisation of single V_H domain antibodies (V_H2sd and V_H4sd) directed against APS.

This example demonstrates that V_H2 and V_H4 variable domains directed against APS (described in Example 3) can bind this antigen in the absence of a complementary variable domain.

DNA preps of the scFv clones V_H2 and V_H4 (described in Example 3) were digested with *NcoI/XhoI* to cut out the V_H domains (Figure 2). These domains were then ligated into a *NcoI/XhoI* digested pIT1 vector (Figure 2) to create V_H single domain fusion with gene III.

The binding characteristics of the produced clones (V_H2sd and V_H4sd) were then tested by monoclonal phage ELISA. Phage particles were produced as described by *Harrison et al.*, (1996). 96-well ELISA plates were coated with 100µl of APS, BSA, HSA, β-gal, ubiquitin, α-amylase and myosin at 10µg/ml concentration in PBS overnight at 4°C. A standard ELISA protocol was followed (Hoogenboom *et al.*, 1991) using detection of bound phage with anti-M13-HRP conjugate. ELISA results demonstrated that V_H single domains specifically recognised APS when displayed on the surface of the filamentous bacteriophage (Figure 9). The ELISA of soluble V_H2sd and V_H4sd gave the same results

as the phage ELISA, indicating that these single domains are also able to recognise APS as soluble fragments (Figure 10).

Example 5. Selection of single V_H domain antibodies directed against APS and single V_K domain antibodies directed against β-gal from a repertoire of single antibody domains.

This example describes a method for making single V_H domain antibodies directed against APS and single V_K domain antibodies directed against β-gal by selecting repertoires of virgin single antibody variable domains for binding to these antigens in the absence of the complementary variable domains.

Two human phage antibody libraries were used in this experiment.

Library 5	NNK V_H single domain	4.08×10^8
Library 6	NNK V_K single domain	2.88×10^8

The libraries are based on a single human framework for V_H (V3-23/DP47 and J_H4b) and V_K (O12/O2/DPK9 and J_K1) with side chain diversity incorporated in complementarity determining regions (CDR2 and CDR3). V_H sequence in Library 5 (complementary V_K variable domain being absent) is diversified at positions H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97 and H98 (NNK encoded). V_K sequence in Library 6 (complementary V_H variable domain being absent) is diversified at positions L50, L53, L91, L92, L93, L94 and L96 (NNK encoded) (Figure 1). The libraries are in phagemid pIT1/single variable domain format (Figure 2).

Two rounds of selections were performed on APS and β-gal using Library 5 and Library 6, respectively. In the case of APS the phage titres went up from 9.2×10^5 in the first round to 1.1×10^8 in the second round. In the case of β-gal the phage titres went up from

2.0×10^6 in the first round to 1.6×10^8 in the second round. The selections were performed as described in Example 1 using immunotubes coated with either APS or β-gal at 100μg/ml concentration.

After second round 48 clones from each selection were tested for binding to their respective antigens in a soluble single domain ELISA. 96-well plates were coated with 100μl of 10μg/ml APS and BSA (negative control) for screening of the clones selected from Library 5 and with 100μl of 10μg/ml β-gal and BSA (negative control) for screening of the clones selected from Library 6. Production of the soluble V_K and V_H single domain fragments was induced by IPTG as described by Harrison *et al.*, (1996) and the supernatant (50μ) containing single domains assayed directly. Soluble single domain ELISA was performed as soluble scFv ELISA described in Example 1 and the bound V_K and V_H single domains were detected with Protein L-HRP and Protein A-HRP, respectively. Five V_H single domains (V_HA10sd, V_HA1sd, V_HA5sd, V_HC5sd and V_HC11sd) selected from Library 5 were found to bind APS and one V_K single domain (V_KE5sd) selected from Library 6 was found to bind β-gal. None of the clones cross-reacted with BSA (Figures 3, 11).

Example 6. Creation and characterisation of the dual specific scFv antibodies (V_KE5/V_H2 and V_KE5/V_H4) directed against APS and β-gal.

This example demonstrates that dual specific scFv antibodies (V_KE5/V_H2 and V_KE5/V_H4) directed against APS and β-gal could be created by combining V_KE5sd variable domain that was selected for binding to β-gal in the absence of a complementary variable domain (as described in Example 5) with V_H2 and V_H4 variable domains that were selected for binding to APS in the presence of the complementary variable domains (as described in Example 3).

To create these dual specific antibodies, pIT1 phagemid containing V_KE5sd (Example 5) was digested with NcoI/XhoI (Figure 2). NcoI/XhoI fragments containing V_H variable domains from clones V_H2 and V_H4 (Example 3) were then ligated into the phagemid to create scFv clones V_KE5/V_H2 and V_KE5/V_H4, respectively.

The binding characteristics of the produced clones were tested in a soluble scFv ELISA. A 96-well plate was coated with 100µl of APS, β-gal and BSA (negative control) at 10µg/ml concentration in PBS overnight at 4°C. Production of the soluble scFv fragments was induced by IPTG as described by Harrison *et al.*, (1996) and the supernatant (50µ) containing scFvs assayed directly. Soluble scFv ELISA was performed as described in Example 1 and the bound scFvs were detected with Protein L-HRP. Both V_KE5/V_H2 and V_KE5/V_H4 clones were found to be dual specific. No cross-reactivity with BSA was detected (Figure 12).

Example 7. Construction of vectors for converting the existing scFv dual specific antibodies into a Fab format.

a. Construction of the C_K vector and Ck/gIII vector.

C_K gene was PCR amplified from an individual clone A4 selected from a Fab library (Griffith *et al.*, 1994) using CkBACKNOT as a 5' (back) primer and CKSACFORFL as a 3' (forward) primer (Table 1). 30 cycles of PCR amplification were performed as described by Ignatovich *et al.*, (1997), except that *Pfu* polymerase was used as an enzyme. PCR product was digested with NotI/EcoRI and ligated into a NotI/EcoRI digested vector pHEN14V_K (Figure 13) to create a C_K vector (Figure 14).

Gene III was then PCR amplified from pIT2 vector (Figure 2) using G3BACKSAC as a 5' (back) primer and LMB2 as a 3' (forward) primer (Table 1). 30 cycles of PCR amplification were performed as above. PCR product was digested with SacI/EcoRI and

ligated into a SacI/EcoRI digested C_K vector (Figure 14) to create a Ck/gIII phagemid (Figure 15).

b. Construction of the C_H vector.

C_H gene was PCR amplified from an individual clone A4 selected from a Fab library (Griffith *et al.*, 1994) using CHBACKNOT as a 5' (back) primer and CHSACFOR as a 3' (forward) primer (Table 1). 30 cycles of PCR amplification were performed as above. PCR product was digested with NotI/BglII and ligated into a NotI/BglII digested vector PACYC4V_H (Figure 16) to create a C_H vector (Figure 17).

Example 8. Construction of V_KE5/V_H2 Fab clone and comparison of its binding properties with the V_KE5/V_H2 scFv version (Example 6).

This example demonstrates that the dual specificity of the V_KE5/V_H2 scFv antibody is retained when the V_K and V_H variable domains are located on different polypeptide chains. Furthermore, the binding of the V_KE5/V_H2 Fab clone to β-gal and APS becomes competitive. In contrast, V_KE5/V_H2 scFv antibody can bind to both antigens simultaneously.

To create a V_KE5/V_H2 Fab, DNA from V_KE5/V_H2 scFv clone was digested with SalI/NotI and the purified DNA fragment containing V_KE5 variable domain was ligated into a SalI/NotI digested C_K vector (Figure 14). Ligation products were used to transform competent *Escherichia coli* TG-1 cells as described by Ignatovich *et al.*, (1997) and the transformants (V_KE5/C_K) were grown on TYE plates containing 1% glucose and 100μg/ml ampicillin.

DNA from V_KE5/V_H2 scFv clone was also digested with SfiI/XhoI and the purified DNA fragment containing V_H2 variable domain was ligated into a SfiI/XhoI digested C_H vector (Figure 17). Ligation products were used to transform competent *E. coli* TG-1 cells

as above and the transformants (V_H2/C_H) were grown on TYE plates containing 1% glucose and 10μg/ml chloramphenicol.

DNA prep was then made form V_KE5/C_K clone and used to transform V_H2/C_H clone as described by Chung *et al.*, (1989). Transformants were grown on TYE plates containing 1% glucose, 100μg/ml ampicillin and 10μg/ml chloramphenicol.

The clone containing both V_KE5/C_K and V_H2/C_H plasmids was then induced by IPTG to produce soluble V_KE5/V_H2 Fab fragments. Inductions were performed as described by Harrison *et al.*, (1996), except that the clone was maintained in the media containing two antibiotics (100μg/ml ampicillin and 10μg/ml chloramphenicol) and after the addition of IPTG the temperature was kept at 25°C overnight.

Binding of soluble V_KE5/V_H2 Fabs was tested by ELISA. A 96-well plate was coated with 100μl of APS, β-gal and BSA (negative control) at 10μg/ml concentration in PBS overnight at 4°C. Supernatant (50μ) containing Fabs was assayed directly. Soluble Fab ELISA was performed as described in Example 1 and the bound Fabs were detected with Protein A-HRP. ELISA demonstrated the dual specific nature of V_KE5/V_H2 Fab (Figure 18).

The produced V_KE5/V_H2 Fab was also purified from 50 ml supernatant using Protein A-Sepharose as described by Harlow & Lane (1988) and run on a non-reducing SDS-PAGE gel. Coomassie staining of the gel revealed a band of 50kDa corresponding to a Fab fragment (data not shown).

A competition ELISA was then performed to compare V_KE5/V_H2 Fab and V_KE5/V_H2 scFv binding properties. A 96-well plate was coated with 100μl of β-gal at 10μg/ml concentration in PBS overnight at 4°C. A dilution of supernatants containing V_KE5/V_H2 Fab and V_KE5/V_H2 scFv was chosen such that OD 0.2 was achieved upon detection with Protein A-HRP. 50μl of the diluted V_KE5/V_H2 Fab and V_KE5/V_H2 scFv supernatants were incubated for one hour at room temperature with 36, 72 and 180μmoles of either

native APS or APS that was denatured by heating to 70°C for 10 minutes and then chilled immediately on ice. As a negative control, 50µl of the diluted $V_K E5/V_H 2$ Fab and $V_K E5/V_H 2$ scFv supernatants were subjected to the same incubation with either native or denatured BSA. Following these incubations the mixtures were then put onto a β-gal coated ELISA plate and incubated for another hour. Bound $V_K E5/V_H 2$ Fab and $V_K E5/V_H 2$ scFv fragments were detected with Protein A-HRP.

ELISA demonstrated that $V_H 2$ variable domain recognises denatured form of APS (Figure 19). This result was confirmed by BIAcore experiments when none of the constructs containing $V_H 2$ variable domain were able to bind to the APS coated chip (data not shown). ELISA also clearly showed that a very efficient competition was achieved with denatured APS for $V_K E5/V_H 2$ Fab fragment, whereas in the case of $V_K E5/V_H 2$ scFv binding to β-gal was not affected by competing antigen (Figure 19). This could be explained by the fact that scFv represents a more open structure where V_K and V_H variable domains can behave independently. Such freedom could be restricted in a Fab format.

All publications mentioned in the above specification, and references cited in said publications, are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

Claims

1. A method for producing a dual-specific ligand comprising a first single immunoglobulin variable domain having a first binding specificity and a complementary immunoglobulin single variable domain having a second binding specificity, the method comprising the steps of:

a) selecting a first variable domain by its ability to bind to a first antigen,
b) selecting a second variable region by its ability to bind to a second antigen,
c) combining the variable regions; and
d) selecting the dual-specific ligand by its ability to bind to said first and second antigens.

2. A method according to claim 1 wherein said first variable domain is selected for binding to said first antigen in absence of a complementary variable domain.

3. A method according to claim 1 wherein said first variable domain is selected for binding to said first antigen in the presence of a third complementary variable domain in which said third variable domain is different from said second variable domain.

4. A method according to claim 2 or claim 3, wherein the first and second antigens compete for binding such that the dual specific ligand may not bind both antigens simultaneously.

5. A method according to claim 2 or claim 3, wherein the first and second antigens bind independently, such that the dual specific ligand may simultaneously bind both the first and second antigens.

6. A method according to claim 4 and 5, wherein the dual specific ligand comprises a first form and a second form in equilibrium in solution, wherein both antigens bind to the first form independently but compete for binding to the second form.

7. A method according to any preceding claim wherein the variable regions are derived from immunoglobulins directed against said antigens.

8. A method according to any preceding claim wherein the variable domain is derived from a repertoire of single antibody domains.

9. A method of claim 8 wherein said repertoire is displayed on the surface of filamentous bacteriophage and wherein the single antibody domains are selected by binding of the bacteriophage repertoire to antigen.

10. A method of any preceding claim wherein the sequence of at least one variable domain is modified by mutation or DNA shuffling.

11. A dual-specific ligand comprising a first single immunoglobulin variable domain having a binding specificity to a first antigen and a second complementary immunoglobulin single variable domain having a binding activity to a second antigen.

12. A dual specific ligand according to claim 11, obtainable by a method according to any one of claims 1 to 10.

13. A dual-specific ligand according to claim 10 or claim 11, comprising at least one single heavy chain variable domain of an antibody and one complementary single light chain variable domain of an antibody such that the two regions are capable of associating to form a complementary VH/VL pair.

14. A dual specific ligand according to claim 13 which comprises an antibody scFv fragment.

15. A dual-specific ligand according to claim 13 which comprises an antibody Fab region.

16. An IgG comprising a dual specific ligand of claim 14.

17. A dual-specific ligand according to any one of claims 11 to 16 wherein the variable regions are non-covalently associated.

18. A dual-specific ligand according to any one of claims 11 to 16 wherein the variable regions are covalently associated.

19. A dual-specific ligand according to claim 18 wherein the covalent association is mediated by di-sulphide bonds.

20. A dual specific ligand of claim 14 which comprises a universal framework.

21. A dual specific ligand of claim 14 which comprises the binding site for a specific generic ligand.

22. A kit comprising a dual-specific ligand according to any one of claims 11 to 21.

23. Nucleic acid encoding at least a dual-specific ligand according to any one of claims 11 to 21.

24. A vector comprising nucleic acid according to claim 23.

25. A vector according to claim 24, further comprising components necessary for the expression of a dual-specific ligand.

26. A host cell transfected with a vector according to claim 25.

Abstract

The invention provides a dual-specific ligand comprising a first single immunoglobulin variable domain having a first binding specificity and a complementary immunoglobulin single variable domain having a second binding specificity.

H1 H10 H20 H30

E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F S

GAG GTG CAG CTG TTG GAG TCT GGG GGA GGC TTG GTA CAG CCT GGG GGG TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTT AGC

H40 H50 H52 a

S Y A M S W V R Q A P G K G L E W V S A I S G S G G S T Y Y

AGC TAT GCC ATG AGC TGG GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTC TCA GCT ATT AGT GGT AGT GGT GGT AGC ACA TAC TAC

HCDR1 HCDR2

H60 H70 H80 H82 a b c

A D S V K G R F T I S R D N S K N T L Y L Q M N S L R A E D

GCA GAC TCC GTG AAG GGC CGG TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT CTG CAA ATG AAC AGC CTG AGA GCC GAG GAC

H90 H98 H100 H110 H113

T A V Y Y C A K S Y G A F D Y W G Q G T L V T V S S G G G G

ACG GCC GTA TAT TAC TGT GCG AAA AGT TAT GGT GCT TTT GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC GTC TCG AGC GGT GGA GGC GGT

HCDR3 XhoI

L1 L10

S G G G G S G G G G S T D I Q M T Q S P S S L S A S V G D R

TCA GGC GGA GGT GGC AGC GGC GGT GGC GGG TCG ACG GAC ATC CAG ATG ACC CAG TCT CCA TCC TCC CTG TCT GCA TCT GTA GGA GAC AGA

SalI

L20 L30 L40

V T I T C R A S Q S I S S Y L N W Y Q Q K P G K A P K L L I

GTC ACC ATC ACT TGC CGG GCA AGT CAG AGC ATT AGC AGC TAT TTA AAT TGG TAT CAG CAG AAA CCA GGG AAA GCC CCT AAG CTC CTG ATC

LCDR1

L50 L60 L70

Y A A S S L Q S G V P S R F S G S G S G T D F T L T I S S L

TAT GCT GCA TCC AGT TTG CAA AGT GGG GTC CCA TCA AGG TTC AGT GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGT CTG

LCDR2

L80 L90 L100 L107

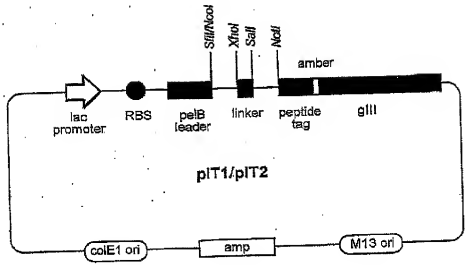
Q P E D F A T Y Y C Q Q S Y S T P N T F G Q G T K V E I K R

CAA CCT GAA GAT TTT GCA ACT TAC TAC TGT CAA CAG AGT TAC AGT ACC CCT AAT ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA CGG

LCDR3

Figure 1

2/19



RBS
CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATA ATG AAA TAC CTA
M K Y L
----------->
LMB3

StiI NcoI
TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCG GCC CAG CCG GCC ATG GCC GAG GTG TTT
L P T A A A G L L L L A A Q P A M A E V F

XhoI linker
GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC GTC TCG AGC GGT GGA GGC GGT TCA GGC GGA GGT
D Y W G Q G T L V T V S S G G G G S G G G

SalI NotI
GGC AGC GGC GGT GGC GGG TCG ACG GAC ATC CAG ATG ACC CAG GCG GCC GCA GAA CAA AAA CTC
G S G G G G S T D I Q M T Q A A A E Q K L
<----------
link seq new

HIS-tag
CAT CAT CAT CAC CAT CAC GGG GCC GCA
H H H H H H G A A
(insertion in pIT2 only)

Gene III
myc-tag
ATC TCA GAA GAG GAT CTG AAT GGG GCC GCA TAG ACT GTT GAA AGT TGT TTA GCA AAA CCT CAT
I S E E D L N G A A * T V E S C L A K P H
<----------
pHEN seq

FIGURE 2

V_H chains

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
	1 2 3		4	5 6	7 8 9	10	11
	123456789012345678901234567890	12345	67890123456789	012a3456789012345	67890123456789012abc345678901234	567801	234567890123
V_H dummy	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK	SYGAFDY	WGQGTLVTVSS
K8	------------------------------	-----	--------------	H-SPY-AN-R-------	--------------------------------	GLRA---	-----------
VH2	------------------------------	-----	--------------	D-GRT-SK-G---P---	--------------------------------	KVLT---	-----------
VH4	------------------------------	-----	--------------	R-NGP-*A-G-------	----------------I---------------	HGAW---	-----------
VHC11	------------------------------	----N	--------------	S-PAS-LH-R-------	--------------------------------	PGLG---	-----------
VHA10sd	------------------------------	-----	--------------	D-ERT-Y*-R-------	--------------------------------	KVLV---	-----------
VHA1sd	------------------------------	-----	--------------	H-SAN-SK-Q-------	-L------------------------------	KVLQ---	-----------
VHA5sd	------------------------------	-----	--------------	T-PAS-*V-R-------	--------------------------------	SLLQ---	-----------
VHC5sd	------------------------------	-----	--------------	D-AAT-SA-S-------	--------------------------------	KILK---	-----------
VHC11sd	----------------------------S-	-----	--------------	T-SSV-QS-R-------	--------------------------------	NLMS---	-----------

V_K chains

	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
	1 2	3	4	5	6 7 8	9	10
	12345678901234567890123	45678901234	567890123456789	0123456	78901234567890123456789012345678	901234567	8901234567 8
V_K dummy	DIQMTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTPNT	FGQGTKVEIKR
K8	-----------------------	-----------	---------------	R--H---	--------------------------------	--PWRS-G-	-----------
E5sd	-----------------------	----V------	---------------	L--R---	--------------------------------	--NWRL-P-	-----------
C3	-----------------------	-----------	---------------	*--L---	--------------------------------	--RVYD-L-	-----------

Figure 3

4/19

1.421
0.08
1.199
0.088
1.338
0.087
0.076

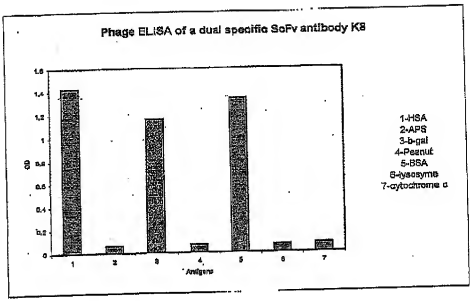


Figure 4

Soluble ELISA of the Dual Specific ScFv Antibody K8
HSA
BSA
b-gal
Protein A
OD
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
600
K8 ScFv concentration (nmol)

Figure 5

0.018
1.422
0.016
1.5

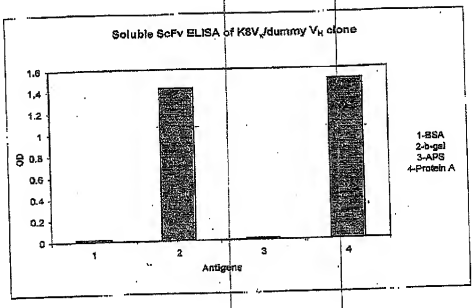


Figure 6

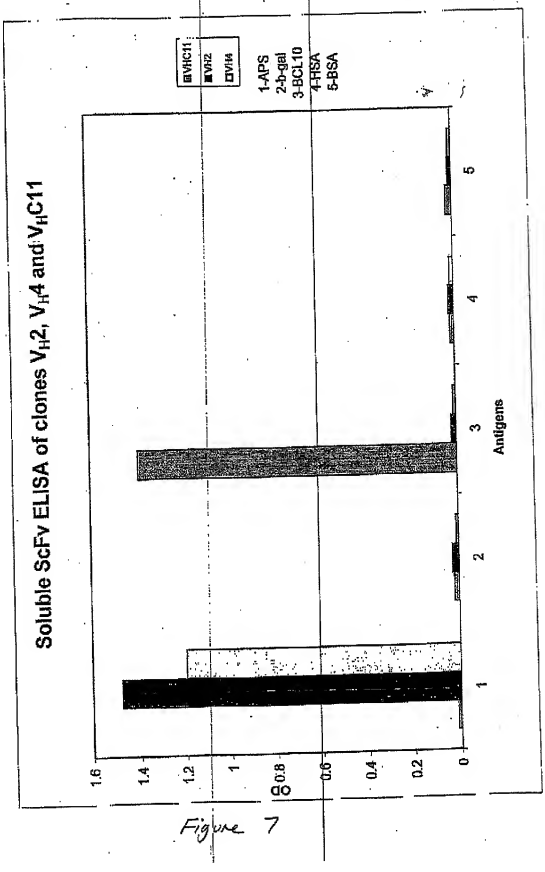

Soluble ScFv ELISA of clones V_H2, V_H4 and V_HC11
VHC11
VH2
VH4
1-APS
2-b-gal
3-BCL10
4-HSA
5-BSA
OD
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
Antigens

Figure 7

8/19

K8Vk/VH2	K8Vk/VH4	K8Vk/VHC11
1.506	1.724	0.017
0.824	1.387	0.947
0.017	0.034	0.804
0.02	0.02	0.033
0.013	0.02	0.052

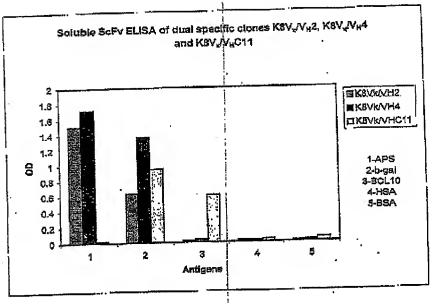


Figure 8

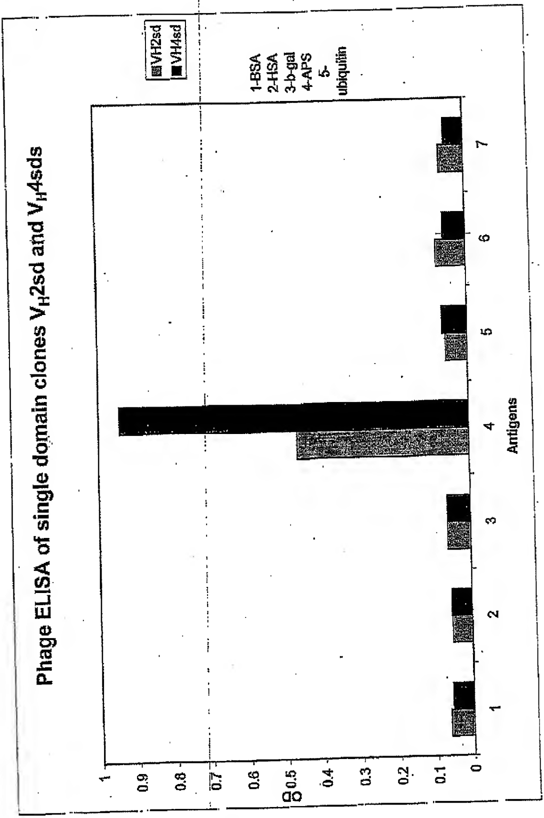

Phage ELISA of single domain clones VH2sd and VH4sds
VH2sd
VH4sd
1-BSA
2-HSA
3-b-gal
4-APS
5-ubiquitin
OD
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1
2
3
4
5
6
7
Antigens

Figure 9

10/19

VH2sd	VH4sd
0.02	0.014
0.035	0.029
0.058	0.049
1.375	1.414
0.022	0.02
0.032	0.034
0.046	0.042

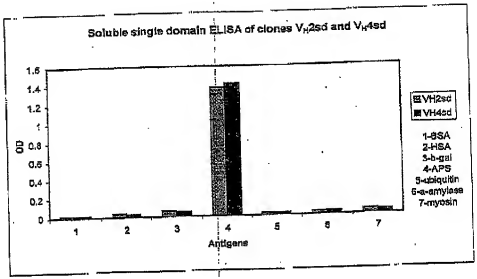


Figure 10

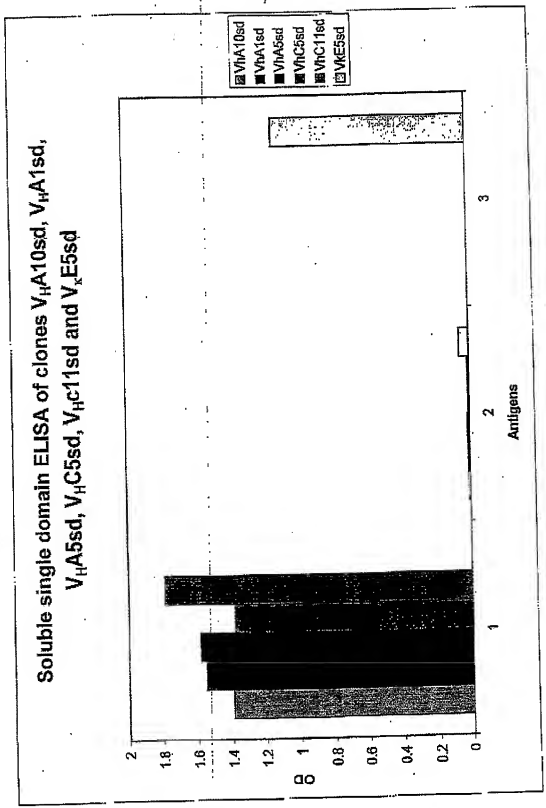

Soluble single domain ELISA of clones V_HA10sd, V_HA1sd, V_HA5sd, V_HC5sd, V_HC11sd and V_KE5sd
VhA10sd
VhA1sd
VhA5sd
VhC5sd
VhC11sd
VkE5sd
OD
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
Antigens

Figure 11

12/19

VkE5/Vh2	VkE5/Vh4
0.014	0.016
0.472	1.018
1.69	2.017

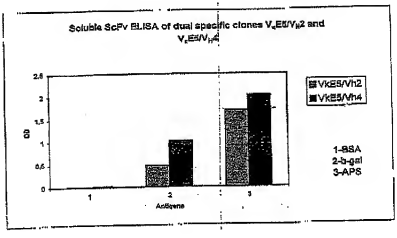
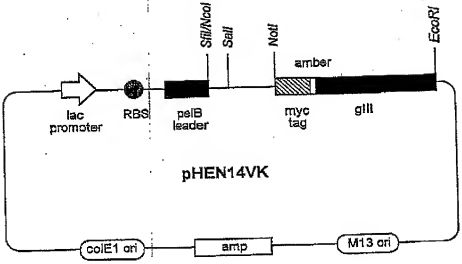


Figure 12



RBS
CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATA ATG AAA TAC CTA
M K Y L

LMB3

Sfil NcoI SalI
TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCG GCC CAG CCG GCC ATG GCG TCG ACG GAC
L P T A A A G L L L L A A Q P A M A S T D

NotI myc tag
ATC CAG ATG ACC CAG GCG GCC GCA GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT GGG GCC
I Q M T Q A A A E Q K L I S E E D L N G A
<----------
pHENseq

gene III
GCA TAG ACT GTT GAA AGT TGT TTA GCA AAA CCT CAT
A O T V E S C L A K P H

Figure 13

14/19

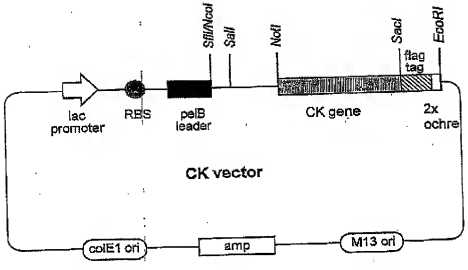


Figure 14

15/19

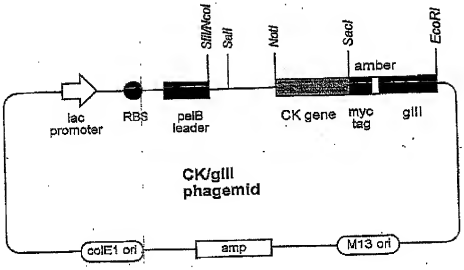
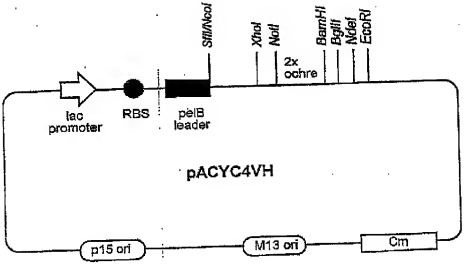


Figure 15



RBS

CAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCAAATTCTATTTCAAGGAGACAGTCATA ATG AAA TAC CTA
M K Y L

LMB3 ------>

SfiI NcoI

TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCG GCC CAG CCG GCC ATG GCC GAG GTG TTT
L P T A A A G L L L L A A Q P A M A E V F

XhoI NotI BamHI BglII

GAC TAC TGG GGC CAG GGA ACC CTG GTC ACC GTC TCG AGC GCG GCC GCA TAA TAA GGA TCC AGA
D Y W G Q G T L V T V S S A A A O O G S R

NdeI EcoRI

TCT CAT ATG GAA TTC
S H M E F

Figure 16

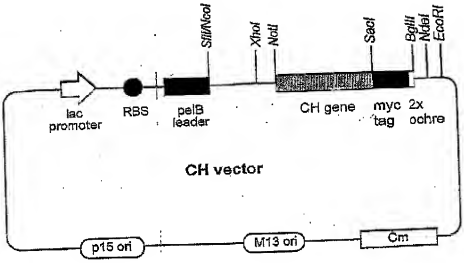

SfiI/NcoI
XhoI
NotI
SacI
BglII
NdeI
EcoRI
lac promoter
RBS
pelB leader
CH gene
myc tag
2x ochre
CH vector
p15 ori
M13 ori
Cm

Figure 17

1.802
1.823
0.03

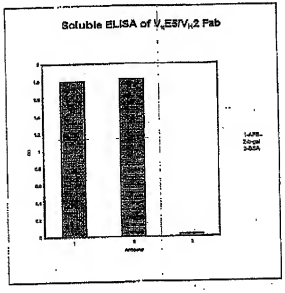
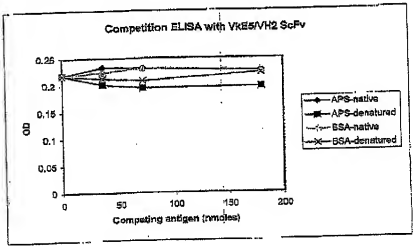


Figure 18

Conc.(nmoles	APS-native	APS-denature	BSA-native	BSA-denatured
0	0.218	0.218	0.218	0.218
36	0.233	0.201	0.224	0.212
72	0.231	0.194	0.231	0.208
180	0.226	0.198	0.227	0.222



Conc. (nmoles	APS-native	APS-denature	BSA-native	BSA-denatured
0	0.227	0.227	0.227	0.227
36	0.225	0.097	0.221	0.22
72	0.225	0.089	0.229	0.216
180	0.231	0.09	0.23	0.213

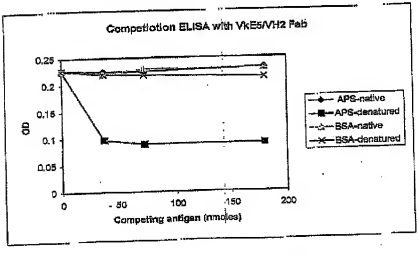


Figure 19

PCT GB 02 03014

24/7/02

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